LyT-1⁺ T Cells Participate in Recovery from Ocular Herpes Simplex Virus Type 1 Infection

John E. Oakes, James T. Rector, and Robert N. Lausch

Herpes simplex virus type 1 (HSV-1) inoculated on the abraded cornea of 4-week-old irradiated (450 R) SJL/J mice spread to the brain and produced a fatal viral encephalitis by 10–12 days post-infection. Adoptive transfer of 2–3 × 10⁷ virus-sensitized non-adherent spleen cells 24 hrs before virus challenge led to recovery from infection. Recovery was due to suppression of HSV-1 replication in the brain rather than prevention of virus spread from the cornea to the central nervous system. If the immune cells were treated with monoclonal anti-Thy 1.2 and complement before transfer the protective effect was lost. Pretreatment of the virus-sensitized spleen cells with monoclonal antibody to the Lyt-1 membrane antigen also significantly reduced or completely abrogated the protective effect. In contrast, depletion of Lyt-2 bearing cells resulted in a less-pronounced and not statistically significant loss of protection. It was further observed that spleen cell reconstituted, virus challenged mice had considerably higher serum antibody titers (>10-fold) than unconstituted virus challenged controls. The results indicate that lymphocytes with a Thy 1.2⁺, Lyt-1⁺, Lyt-2⁻ phenotype play a predominant role in promoting recovery following corneal HSV-1 infection. Invest Ophthalmol Vis Sci 25:188–194, 1984

Previous studies in our laboratory have shown that mice treated with herpes simplex virus type 1 (HSV-1) specific antibody following corneal inoculation do not develop fatal herpetic encephalitis. Treatment of animals with antithymocyte serum prior to passive transfer of antibody abrogated the protective effect. Therefore, we speculated that antibody may be slowing virus spread within the central nervous system (CNS) until a cellular immune response could be initiated. The important role T lymphocytes play in resolving subcutaneous and intraperitoneal HSV-1 infections has been extensively documented in adoptive transfer experiments, but the role of T cell subsets in ocular HSV-1 infection has not been investigated. The complexity of the T lymphocyte response following antigen stimulation has recently become recognized. Three T cell subsets designated Lyt-1⁺, Lyt-2⁻, and Lyt-1⁻2⁻ have been identified in mice. The Lyt-1⁺ cells amplify B-cell responses and mediated delayed-type hypersensitivity (DTH) reactions while Lyt-2⁻ cells are involved in cytotoxic T-cell lysis (CTL). Cells with the Lyt-1⁺ 2⁻ phenotype are generally believed to be the precursors to the other two types.

The present study was initiated in order to examine what role T cells and their subsets play in resolving ocular herpes virus infection. We found that adoptive transfer of HSV-1 sensitized T cells prior to corneal HSV-1 infection did not prevent virus spread to the brain but did resolve the infection following virus invasion of the CNS. Furthermore, T cells with a Lyt-1 phenotype were the dominant mediator of the antiviral protection seen in immune spleen cell reconstituted recipients.

Materials and Methods

Preparation of Virus

HSV-1 (strain KOS), originally obtained from Dr. Fred Rapp, Hershey, PA, has been maintained in this laboratory by passage on Vero cells (Flow Laboratories, Rockville, MD) at a multiplicity of infection of 0.1 PFU/cell. Growth medium consisted of minimal essential medium (MEM) supplemented with antibiotics, 5% newborn calf serum and sodium bicarbonate. Virus was harvested from infected cells by three cycles of freezing and thawing. The lysate was clarified by centrifugation at 1,000 × g for 10 min and assayed on Vero cells for virus titer as previously described.

Infection of Mice

SJL/J mice (Jackson Labs., Bar Harbor, Maine) were anesthetized with 0.004 mg of pentobarbital/g of body
weight. The right eye was then scarified by three twists with a corneal trephine. A volume of 0.01 ml of MEM containing approximately 3-4 x 10⁷ PFU of HSV-1 was dropped onto the surface of the cornea and massaged into the eye with eyelid. The pathogenesis of HSV-1 in mice following ocular infection in this manner has been described.¹⁵

Preparation of Immune Spleen Cells

Immune spleen cell donors were immunized subcutaneously in the right rear footpad with 1 x 10⁶ PFU of HSV. Three weeks later, the right rear footpads were injected with approximately 0.05 ml of 10% saline. The edematous areas were abraded with an emory board 6 hrs later to remove the stratum corneum. One drop of virus suspension from a 26-gauge needle (2 x 10⁶ PFU/ml) was placed on the abraded area and rubbed in with the shank of the needle. Control mice were treated with Hank’s basal salt solution (HBSS) in place of virus. Six days later, spleens were removed from donors and extruded aseptically from their capsules. After being minced with scissors, the material was filtered through sterile cotton gauze to remove clumps of tissue. Cells were washed and resuspended in HBSS and counted. In some experiments, adherent cells were depleted by placing 15 ml of the suspension (1 x 10⁷ cells/ml) in 150 cm² tissue culture flasks at 37°C in 5% CO₂ for 4 hrs. Nonadherent cells were then decanted after gentle rotation of the flask.

Adoptive Transfer of Immunity

Mice were immunosuppressed with 450 R whole-body irradiation in groups of 12 in a square Lucite box 30 cm from the target of a 100 kv power x-ray source (model no. 100/1, General Electric, Milwaukee, Wis.) operated at a tube current of 5mA with a 2-in aluminum filter. The exposure dose rate delivered was 37.6 rad/min as measured by a Victoreen R-meter (Instr. Div., Victoreen, Cleveland, OH). Following irradiation, spleen cells were transferred to irradiated mice by intravenous inoculation. Twenty-four hours later, mice were infected ocularly as described above.

Assay of Tissues for HSV-1

Eye globes, trigeminal ganglia and brains were dissected out of animals and homogenized in a Ten Broeck homogenizer (Belco, Vineland, NH) containing MEM with 5% newborn calf serum to give a 10% suspension. The tissues were then frozen and thawed three times and centrifuged at 1000 x g for 10 min at 22°C. The supernatants were then used for virus assays on monolayers of Vero cells.

Depletion of T-lymphocytes and T-lymphocyte Subsets

HSV-1 sensitized spleen cells (10 x 10⁸ cells/ml) were incubated at 37°C with a 1:5 dilution of monoclonal anti-Thy 1.2 (New England Nuclear, Boston, MA) and 1:2 dilution of low toxicity rabbit complement (C) (Cedarlane Laboratories Limited, Hicksville, NY) for one hr. The cells were washed and the treatment repeated. The cells were then washed twice and adjusted to the desired concentration.

Spleen cells were depleted of Lyt-1 and/or Lyt-2-bearing cells by treatment with monoclonal anti-mouse Lyt-1 and/or anti-mouse Lyt-2 arsanilate conjugates (Becton Dickinson, Sunnyvale, CA). Cytotoxic titers were 0.1 µg and 0.03 µg per 3 x 10⁵ lymph node cells, respectively. Spleen cells (3-7 x 10⁸) were incubated with 50 µl of the desired undiluted conjugate for 1 hr at 4°C. The cells were then washed and incubated with rabbit anti-arsanilate (50 µl) for 30 min at 4°C. Then, 0.2 ml C was added for 30 min at 37°C. The cells were washed twice and adjusted to the desired concentration before transfer to recipients. In some experiments, treatment of spleen cells with anti-Lyt serum was repeated a second time before transfer.

Elisa Test for Anti HSV-1 Antibody

Elisa tests were carried out in Linbro tissue culture plates (Cat. no. 76-003-05; Flow Laboratories, McLean VA). HSV-1 infected or noninfected Vero cells were attached to plates as previously described¹⁶ and stored at −20°C until used. Test and control sera were diluted 1:100 and 1:1000 in PBS, pH 7.2, containing 0.05% Tween 20 (Fischer Scientific Co., Pittsburgh, PA), 2mM 2-mercaptoethanol, 100 µg/ml BSA (Sigma, St. Louis, MO) and 0.05% sodium azide. Fifty µl of sera was added to wells and incubated for two hrs at room temperature with constant shaking on a Tektator V (American Scientific Products, New Orleans, LA). Plates were washed four times in PBS, pH 7.2, containing 0.05% Tween 20 and 100 µg/ml BSA. Hybridoma screening reagent (BRL, Gaithersburg, MD) was diluted 1:200 with PBS, containing 0.05% sodium azide, and 50 µl was added to each well and incubated for 2 hrs at room temperature with constant shaking. Plates were then washed as before. Substrate solution was prepared by making a 1.0 mg/ml o-nitrophenyl-B-D-galactoside (Sigma, St. Louis, MO) solution in 50 mM sodium phosphate buffer (pH 7.0) containing 1.5 mM MgCl₂ and 100 mM 2-mercaptoethanol. Fifty µl of substrate were added to each well and incubated for 1 hr at room temperature. Twenty-five µl of 0.5 M sodium carbonate were added to each well to stop the reaction, and the absorbancy at 414 nm was read with a Titertek Multiscan (Flow Labo-
The titer was expressed as the reciprocal of the highest dilution giving a positive reaction. A reaction was defined as positive when it gave at least twice the reading of the uninfected cell control. Known positive and negative control sera were included in each assay. Positive control serum was taken from SJL/J mice hyperimmunized with HSV-1, while negative control serum came from nonimmune SJL/J mice.

Measurement of Neutralizing Antibody

Equal volumes of sera diluted as desired were added to tubes containing 0.2 ml of a HSV-1 suspension (3 × 10³ PFU/ml). These virus-serum mixtures were incubated for 30 min at 37°C, then 0.1-ml aliquots were placed in 60-mm plastic Petri dishes (done in duplicate) containing Vero monolayers.17 Controls received virus only. After adsorption for 1 hr at room temperature, the monolayers were overlaid with 4 ml of 0.5% methylcellulose solution and incubated for 2 days at 37°C in a 5% CO₂ atmosphere. Plaques were then counted after staining with 0.1% crystal violet. The neutralizing titer was taken as that dilution of serum that reduced the mean PFU by 50% in comparison with the controls.

Statistical Analysis of Data in Virus Challenge Experiments

Negative-exponential transformation of survival times (α = 0.1; T = 20) were performed in the virus challenge experiments as described by Liddell.18 The means and variances of survival times for each group were then calculated from the transformed data, and the level of significance between survivors in control groups and survivors in experimental groups was determined by Student's t test. A P value <0.05 was considered significant.

Results

Effect of HSV-1 Sensitized Spleen Cells on Virus Spread in Vivo

Experiments were initiated to assess the effect of immune spleen cells preparations on the course of HSV-1 ocular infection. To investigate this question, 48 mice were infected on the cornea with HSV-1. Twenty-four hours before infection, 24 of the animals were each given 5 × 10⁷ HSV-1 sensitized spleen cells. The remaining 24 animals were left untreated. All 48 animals were then infected on the cornea with HSV-1 24 hr later. At selected times postinfection, mice from both groups were killed and pertinent tissues assayed for virus. Figure 1 shows that during the first 6 days postinfection, the kinetics of virus growth in and spread from the eyes to the brain were similar in all HSV-1 infected mice, regardless of whether they had received HSV-1 sensitized cell preparations or were left untreated. However, by day 8 postinfection, the quantity of virus in tissues of cellular recipients was declining, while the amount of infectious virus was increasing in tissues of untreated mice. By day 10 postinfection, all untreated mice were dead, while tissues from immune cell reconstituted recipients were found to be free of infectious virus. The results indicated that the adoptively transferred cells did not prevent corneal infection nor spread of the virus to the CNS. However,
the cells could mediate a response that abrogated virus growth within the brain as well as in the eye and trigeminal ganglion.

Abrogation of the Protective Activity of HSV-1 Sensitized Spleen Cells with Monoclonal Anti-Thy 1.2 or Anti-Lyt Sera

We next sought to identify the effector cell(s) in the immune spleen cell population that was mediating the protective effect. It was found that removal of adherent cells from the HSV-1 sensitized spleen cell populations had no effect on the ability of sensitized cells to adoptively transfer protection against corneal HSV-1 infection (Fig. 2). However, pretreatment of effector cell populations with either anti-Thy 1.2 (Fig. 2) or anti-Lyt (Table 1) in the presence of complement before adoptive transfer abrogated their ability to protect recipients against subsequent virus challenge. Thus, lymphocytes that possess both Thy-1.2 and Lyt markers transfer resistance to ocular HSV-1 infection.

Lyt Phenotype of T Cells Mediating Immunity to Corneal HSV-1 Infection

To determine the Lyt phenotypes of T-cells conferring resistance against HSV-1 replication, immune spleen cell preparations were depleted of Lyt-1 or Lyt-23 bearing cells prior to adoptive transfer into irradiated recipients. Twenty-four hrs after transfer, the animals were infected on the cornea with HSV-1. The results of a representative experiment are depicted in Figure 3. It was found that treatment of effector cells before transfer with anti-Lyt-1 plus complement significantly abrogated \( P < 0.01 \) their capacity to provide protection, while anti-Lyt-2 and C did not significantly abrogate protection \( P > 0.05 \). Experiments in which spleen cells were treated twice with undiluted anti-Lyt-2 plus complement prior to transfer also failed to significantly abrogate the protective effect (data not shown). The results suggest that cells with Lyt-1 markers are the principle T-cell subset mediating resistance to ocular HSV-1 infection.

Role of Antibody in Host Resistance to Corneal HSV-1 Disease

To investigate the possibility that immune spleen preparations possessing Lyt-1+ cells might have facilitated HSV specific antibody synthesis in HSV-1 infected recipients, sera from irradiated and spleen-cell reconstituted hosts were collected at intervals postinfection and assayed for antibody by ELISA and virus neutralization tests. Sera were also collected from unconstituted HSV-1 infected mice and titered for HSV-1 specific antibody. The results are shown in Table 2.

It was found that HSV-1 specific antibodies were readily detected in sera of spleen-cell reconstituted recipients by day 6 postinfection and continued to increase through day 10 postinfection. In contrast, HSV-1 specific antibodies did not appear in unreconstituted animals infected with HSV-1 until day 7 postinfection, and at day 8–10 postinfection the amount of antibody was at least 10-fold less in these animals than in immune spleen cell recipients. When HSV-1 immune spleen cells were given to irradiated uninfected animals, there was no detectable antibody synthesized as measured by ELISA. These results suggest that adoptively

Table 1. Effect of monoclonal anti-lyt plus complement (c) treatment on protective activity of HSV-1-sensitized spleen cells

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Spleen cell treatment</th>
<th>Number cells transferred</th>
<th>Survival*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C only</td>
<td>2 \times 10^7</td>
<td>6/10 (60%)</td>
</tr>
<tr>
<td></td>
<td>Anti-Lyt-1 and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-Lyt-2 + C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C only</td>
<td>2.5 \times 10^7</td>
<td>8/10 (80%)</td>
</tr>
<tr>
<td></td>
<td>Anti-Lyt-1 and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-Lyt-2 + C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Twenty-four hours after spleen cell transfer, mice were challenged by rubbing one drop of 2 \times 10^9 PFU/ML HSV-1 into one trephine scarified cornea. Animals were observed for 4 weeks.
We have investigated the surface antigen phenotype of immune spleen cells that can promote recovery from ocular HSV-1 infection. The experimental approach was to deplete effector cell populations of Thy 1.2, Lyt-1\(^+\), or Lyt-2\(^+\) bearing cells by treatment with specific monoclonal antibody plus complement. The protective effect of the surviving cells was then examined in adoptive transfer studies. It was found that depletion of Thy 1.2\(^+\) bearing cells abrogated protection confirming that T cell-mediated immunity plays a major role in recovery from ocular HSV-1 infection.\(^2\) Depletion of Lyt-1 bearing cells also resulted in significant loss of the protective activity. On the other hand, depletion of Lyt-2 bearing cells consistently failed to significantly abrogate the protective activity. The latter results are unlikely to be due to insufficient antibody treatment because (a) the cytotoxic titer of the anti-Lyt-2 antiserum was 3-fold higher than the anti-Lyt-1 cytotoxic titer and (b) double treatment with undiluted anti Lyt-2 plus complement still failed to significantly abrogate resistance. The failure of anti-Lyt-2 plus C treatment to significantly abrogate adoptive immunity suggests that Lyt-1\(^+\)2\(^+\) cells; rather than Lyt-1\(^+\)2\(^+\) or Lyt-1\(^+\)2\(^-\) cells are the principal T cell subpopulation mediating protection.

The foregoing results are in agreement with the findings of other investigators. Thus, Nagafuchi et al.\(^1\) in carrying out adoptive transfer studies in the nude mouse concluded that the essential T cell subset providing protection against intracutaneous HSV-1 challenge was of the Lyt 1\(^+\)2\(^-\) phenotype. Similarly, Nash and Gell\(^1\) have recently reported that the T cell subset responsible for suppression of virus replication in the ear was Lyt 1\(^+\)2\(^+\).

If the Lyt 1\(^+\) T cell set plays a dominant role in promoting recovery from HSV-1 ocular-initiated infection what is the protective mechanism(s) involved? Lyt-1\(^+\) cells are known to be involved in DTH reactions to HSV-1 in mice\(^1\) and adoptive transfer of DTH with HSV-sensitized lymphoid cells is well established.\(^1\)\(^0\)\(^2\)\(^0\)\(^2\)\(^1\) In our experiments, mice were given immune cells 24 hrs before virus challenge. Viral assays of tissues from reconstituted mice established that transferred immune cells did not prevent virus spread from sites of infection to the CNS. Rather, the transferred cells mediated some type of immune responses(s) which resulted in the disappearance of virus from infected tissues on or about day 8 postinfection. Therefore, an attractive explanation for our results is that a cell-mediated reaction of the delayed hypersensitivity type was responsible for the disappearance of virus. However, adoptively transferred immune cells may not have completely cleared virus from the host. It is well known that HSV-

### Table 2. Presence of anti-HSV-antibody in sera of irradiated mice given HSV-1-sensitized spleen cells followed by HSV-1 challenge*

<table>
<thead>
<tr>
<th>Number spleen cells transferred</th>
<th>Day bled</th>
<th>HSV-1</th>
<th>ELISA</th>
<th>HSV neutralization</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3 \times 10^7)</td>
<td>3</td>
<td>+</td>
<td>Neg.†</td>
<td>Neg.‡</td>
</tr>
<tr>
<td>(3 \times 10^7)</td>
<td>6</td>
<td>+</td>
<td>1:1000</td>
<td>1:10</td>
</tr>
<tr>
<td>(3 \times 10^7)</td>
<td>8</td>
<td>+</td>
<td>1:10,000</td>
<td>1:100</td>
</tr>
<tr>
<td>(3 \times 10^7)</td>
<td>10</td>
<td>+</td>
<td>ND</td>
<td>1:1000</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>+</td>
<td>Neg.</td>
<td>Neg.</td>
</tr>
<tr>
<td>None</td>
<td>7</td>
<td>+</td>
<td>Neg.</td>
<td>1:10</td>
</tr>
<tr>
<td>None</td>
<td>8</td>
<td>+</td>
<td>1:100</td>
<td>1:10</td>
</tr>
<tr>
<td>None</td>
<td>10</td>
<td>+</td>
<td>1:1000</td>
<td>1:10</td>
</tr>
<tr>
<td>(5 \times 10^7)</td>
<td>7</td>
<td>-</td>
<td>Neg.</td>
<td>Neg.</td>
</tr>
</tbody>
</table>

* SJL/J mice were irradiated (450R). Then a portion of the animals received immune spleen cells. Twenty-four hours later one cornea of all mice was infected with HSV-1 (2 \( \times 10^9 \) PFU/ml). Serum samples (pool of two to three donors) were collected on the days indicated postinfection and assayed for HSV antibody.
† Neg: less than 1:100.
‡ Neg: less than 1:10.
ND: not done.

**Discussion**

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I can establish latent infections in both the trigeminal ganglion and brain. Thus, the immunological reactions mediating by transferred cells may have reduced the amount of infectious virus to undetectable levels by creating an environment that favors the existence of HSV-1 in an inactivated or latent form within the CNS.

Alternatively, or in addition to DTH activity, Lyt-1+ cells could have promoted recovery from HSV-1 infection by amplifying HSV specific antibody response in virus infected recipients. Lyt-1+ cells are known to collaborate with B cells in the generation antibody. In the present study, we observed that a rapid rise in antibody titer was correlated with simultaneous clearance of virus from eye globe, trigeminal ganglion, and brain. A comparable rise in antibody titer was not seen in HSV-1 infected mice not reconstituted with immune cells. Cloning experiments have indicated that a Lyt-1+ T helper cell line can participate in both DTH and antibody generation.

Finally, it is possible that Lyt-1+ cells directly lysed HSV-infected cells. This seems unlikely for several reasons. Lytic activity is usually associated with Lyt-23+ cells, and SJL/J HSV-1 sensitized spleen cell preparations failed to lyse HSV-infected syngeneic or xenogeneic fibroblastic target cells when tested in 51chromium release assays (personal observation). In addition, the consistent failure of several laboratories to detect cytotoxic T cells in HSV-sensitized mice in the absence of in vitro activation also argues against the prospect that direct lysis of virus infected cells by Lyt-1+ T cells was the principal protective mechanism. Whether the release of interferon by HSV-sensitized immune T cells was of critical importance to host recovery remains to be determined.

In conclusion, our studies show that adoptive transfer of immunity to mice infected ocularly with HSV-1 involves cells of a Lyt-1 phenotype. This suggests that T cells mediating DTH and/or antibody synthesis, as opposed to cells with lytic activity, are the predominant initiator of immunological events leading to cessation of virus infection.

Key words: HSV-1, T cell, Lyt-1 lymphocyte, Lyt-23 lymphocyte, monoclonal antibody

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References


